Cored Tubules are Present in Human Epidermal Langerhans Cells

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Cored tubules are ultrastructural organelles described to date only in murine cells belonging to the Langerhans cell family and located in the dermis and its draining lymph nodes. These organelles, the function of which is unknown, differ from Birbeck granules and are interestingly not found in murine epidermal Langerhans cells. In this work we demonstrate that cored tubules are present in freshly isolated human epidermal Langerhans cells. The tubules were found to be interconnected with structures known to belong to the early endosomal pathway and could be immunolabeled with gold-conjugated anti-CD1a and anti-Langerin monoclonal antibodies, but only at 37°C. At this temperature such antibodies are able to progress from the early sorting endosomes to the early recycling endosomes, which in human Langerhans cells include the Birbeck granules. These findings strongly suggest that cored tubules form part of the early recycling compartment.
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Langerhans cells are the representatives of the dendritic cell lineage in the epidermis. These cells have the unique ability to capture and process antigens, leave the epidermis, and migrate through the dermis to the afferent lymph nodes, where they present processed antigens to naive T cells and thus initiate specific immunity (Kripke *et al*, 1990; Moll *et al*, 1993). Langerhans cells are the only epidermal cells to constitutively express major histocompatibility complex class II molecules (Klareskog *et al*, 1977; Rowden *et al*, 1977), CD1a molecules (Fithian *et al*, 1981), and Langerin (Valladeau *et al*, 2000) at their surface. At the ultrastructural level, Langerhans cells also differ from other dendritic cells by the presence of Birbeck granules–distinctive rod-shaped structures of variable length with a central, periodically striated lamella (Birbeck *et al*, 1961).

An additional "specific" ultrastructural organelle called the cored tubule has been reported in mice (Kobayashi and Hoshino, 1978). It is absent from epidermal Langerhans cells but present in the cytoplasm of cells that are thought to belong to the Langerhans cell family and are found in the dermis and its draining lymph nodes (Kobayashi and Hoshino, 1983; Sonoda *et al*, 1985; Bucana *et al*, 1992; 1994). A cored tubule displays a rod-shaped profile when sectioned parallel to its long axis, a circular profile when sectioned transversely, and a "core" appearing as a thin line with no periodicity on longitudinal sections or as a dot on cross-sections. These tubules occur either isolated or grouped in the cytoplasm and, when isolated, tend to run in parallel layers and by branching or anastomozing form irregular networks, which often surround spheroid bodies. Sometimes cored tubules seem to be continuous with vacuoles or the rough endoplasmic reticulum but none appear to be connected to the cell membrane.

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The murine cells "related" to Langerhans cells found in the dermis and its draining lymph nodes contain either cored tubules or Birbeck granules. Intradermal injection of a saline solution, however, induces the appearance of both structures in the same cells, although the Birbeck granules and cored tubules nevertheless remain "scattered independently in the cytoplasm" (Kobayashi and Hoshino, 1983). It is noteworthy that, even after saline injection, cored tubules are not observed in epidermal Langerhans cells, whereas in these cells the number of Birbeck granules increases temporarily 10–30 min later (Kobayashi and Hoshino, 1983).

In this work, we demonstrate for the first time that cored tubules are present in freshly isolated human epidermal Langerhans cells containing Birbeck granules. The relationship of these structures to Birbeck granules, Langerin, and CD1a molecules is discussed and likewise their involvement in the early recycling endosomal compartment of Langerhans cells.

MATERIALS AND METHODS

Antibodies and reagents DCGM4 (IgG1, recognizing an extracellular epitope of Langerin) (Valladeau *et al*, 2000) and BL6 (IgG1, anti-CD1a) were both provided by Immunotech (Marseille, France). Gold-conjugated Fab fragments of the anti-CD1a monoclonal antibody BL6 (BL6-Au) for electron microscopy were obtained from Aurion (Wageningen, The Netherlands). The anti-Langerin monoclonal antibody DCGM4 was labeled with 10 nm gold particles (Goldsols EM-10, Aurion) (DCGM4-Au) as previously reported (Hanau *et al*, 1987). Horseradish peroxidase (HRP) type II (P-8250), diaminobenzidine (DAB) (D-5905), and brefeldin A (BFA) (B-7651) were from Sigma Chemical (St. Louis, MO).

Preparation of epidermal cells Cell suspensions were prepared from normal human skin taken from patients undergoing abdominal plastic surgery, as previously described (Hanau *et al*, 1987). These suspensions initially contained 0.5%–2% freshly isolated Langerhans cells and were enriched in Langerhans cells by gradient centrifugation on Lymphoprep (Flobio, Courbevoie, France), resulting in a Langerhans cell yield of typically 30%–50% of the final cell population.

Immunoelectron microscopy Freshly isolated epidermal cells were either cooled to 19.5°C for 15 min and incubated at 19.5°C for 60 min or warmed to 37°C for 15 min and incubated at 37°C for 25 min with BL6-Au or the gold-conjugated anti-Langerin monoclonal antibody DCGM4 (final dilution 1%), centrifuged at room temperature, and fixed for electron microscopy. Fixation was initiated by adding an equal volume of fixative solution, previously warmed to 37°C, to the cell suspension. The fixative solution contained 3% glutaraldehyde (Electron Microscopy Sciences, Euromedex, Strasbourg, France) and 2% sucrose in 0.1 M sodium cacodylate buffer (both Merck, Darmstadt, Germany) (305 mOsm, pH 7.3). After 5 min the mixture was centrifuged, the supernatant was discarded, and the pellet was resuspended in the same fixative solution and further fixed for 45 min at 37°C. The cells were then washed in 0.1 M sodium cacodylate buffer and postfixed for 1 h at 4°C with 1% osmium tetroxide (Merck) in the same buffer. After further washing in 0.1 M sodium cacodylate buffer, the cells were dehydrated in graded (50%, 70%, 80%, 95%, and 100%) ethanol solutions, incubated overnight in Epon (Electron Microscopy Sciences): absolute alcohol (1 : 1, vol/vol), and embedded in Epon. Ultrathin sections, stained with lead citrate (Leica, Bron, France) and uranyl acetate (Merck), were examined under a Philips CM 120 BioTwin electron microscope (120 kV).

In another series of experiments, freshly isolated epidermal cells were incubated at 37°C for 30 min with BFA (10 μg per ml) and then for 60 min with BFA (10 μg per ml) and HRP (10 mg per ml). The cells were subsequently processed according to Tooze and Hollinshead (1991) and Griffiths *et al* (1989). After washing in phosphate-buffered saline and fixation with 0.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.3, for 30 min at room temperature, the cells were washed in cacodylate buffer and incubated with DAB for 1 min. The HRP–DAB reaction was initiated by adding H_2O_2 to a final concentration of 0.01%. After 30 min in the dark, the reaction was terminated by washing several times in cacodylate buffer and the cells were postfixed in 1% osmium tetroxide, washed again in cacodylate buffer, and further processed as for conventional electron microscopy. Thick and ultrathin sections were examined under a Philips CM 120 BioTwin electron microscope (120 kV), in most cases without poststaining to allow better differentiation of the HRP–DAB reaction product.

RESULTS

During electron microscopy studies of the traffic of CD1a and Langerin molecules in freshly isolated human epidermal Langerhans cells (Salamero *et al*, 2001), we observed two distinctive types of BL6-Au^+ and DCGM4-Au^+ rod-shaped organelles, but only when the cells were incubated at 37°C. First, we observed the "classical" Birbeck granules (**Fig 1**), which appeared as rod-shaped "rigid" structures of variable length with a central, periodically striated lamella. On some sections these structures did not in fact appear rod-shaped but rather disk-shaped, and obliquely or tangentially cut Birbeck granules exhibited a cross-striated pattern or square-lattice appearance, due to the unique arrangement of the particles composing the central lamella. When Birbeck granules were immunogold-labeled with BL6-Au or DCGM4-Au, the gold particles accumulated in their central striated zone, though less with the anti-Langerin antibodies as the level of expression of these molecules is weaker than that of the CD1a molecules at the Langerhans cell surface (data not shown).

Second, we observed some much more tortuous rod-shaped structures with an inner central line lacking periodicity, which when sectioned transversely appeared circular with a central dot (**Fig 2**). These organelles therefore possessed all the ultrastructural characteristics of the "cored tubules" described in mice. Often remaining isolated in the cytoplasm, they were more or less rectilinear and of variable length. These structures could also be continuous, however, with electron-lucent compartments of variable size suggestive of early endosomes, and, by branching or anastomozing, could form pericentriolar networks (**Fig 3*A, B***). Interestingly, when the cored tubules were immunogold-labeled with BL6-Au (**Figs 2, 3**) or with DCGM4-Au (data not shown), the gold particles were found to be localized on their inner central line. Hence, Birbeck granules and cored tubules can coexist in the cytoplasm of Fi Langerhans cells. Although these two rod-shaped organelles were usually scattered independently, we sometimes observed continuity between the tubular elements of the cored tubules and the characteristic pentalaminar structures of Birbeck granules (**Fig 2*A***).

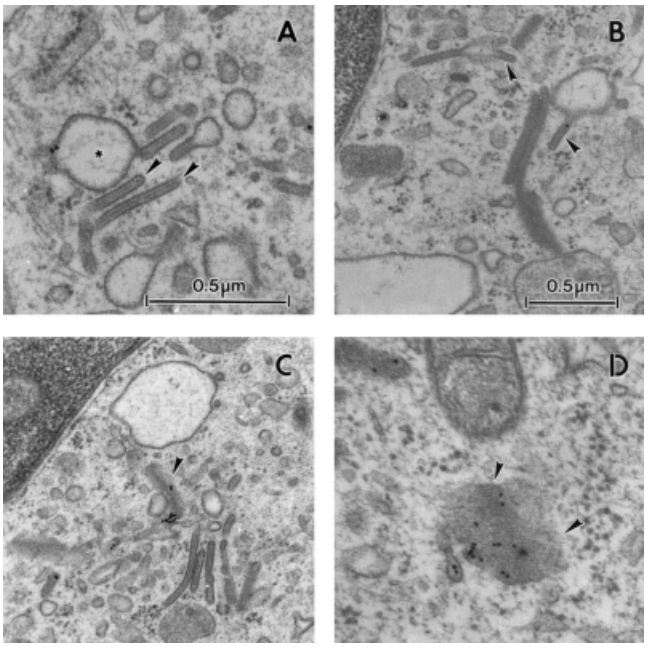

Figure 1. Different aspects of Birbeck granules in freshly isolated human epidermal Langerhans cells. Freshly isolated Langerhans cells were warmed to 37°C for 15 min and incubated at 37°C for 25 min with gold-conjugated Fab fragments of the anti-CD1a monoclonal antibody BL6. In (*A*) the Birbeck granules (*arrowheads*) appear as rod-shaped structures of variable length with a central, periodically striated lamella and lie close to a gold-labeled electron-lucent compartment (*) suggestive of an early endosome. In (*B*) and (*C*), depending on the sections, the Birbeck granules (*arrowheads*) appear as rod-shaped structures or exhibit a cross-striated pattern more or less evident according to whether the plane of the section cuts the granule more obliquely or tangentially. Note the presence of gold particles in the central lamella of the Birbeck granules. In (*D*) the plane of the section passes through the central striated zone of a Birbeck granule (*arrowhead*), allowing visualization of the square-lattice appearance of the central lamella and appreciation of the disk-shaped form of the granule. The inhomogeneous distribution of the gold particles present in the central striated zone should be noted.

Small G proteins of the Arf (ADP-ribosylation factor) family recruit and assemble protein complexes, leading to the formation of cellular coats. This requires their interaction with Arf exchange factors, which catalyze the exchange of GDP for GTP, a process that can be inhibited by BFA (Donaldson *et al*, 1992a; 1992b). Due to its inhibitory effect on coat formation, BFA can induce fusion and redistribution of the membranes of the early endocytic pathway and the *trans* Golgi network, resulting in the formation of continuous tubular networks (Lippincott-Schwartz *et al*, 1991; Wood *et al*, 1991). In Langerhans cells, where coated structures predominate in the pericentriolar area, BFA promotes interconnections between Birbeck granules and the tubular/vacuolar components of the early endosomal system (McDermott *et al*, 2002). When freshly isolated Langerhans cells were treated at 37°C for 30 min with BFA and then for 60 min with BFA and HRP, we likewise observed interconnections between vacuolar early endosomes and cored tubules (**Fig 4**). Thus, continuity between cored tubules and elements of the early endosomal pathway seems to occur spontaneously and is enhanced in the presence of BFA.

DISCUSSION

In this work, we show that cored tubules exist at the periphery and in the central and pericentriolar regions of freshly isolated

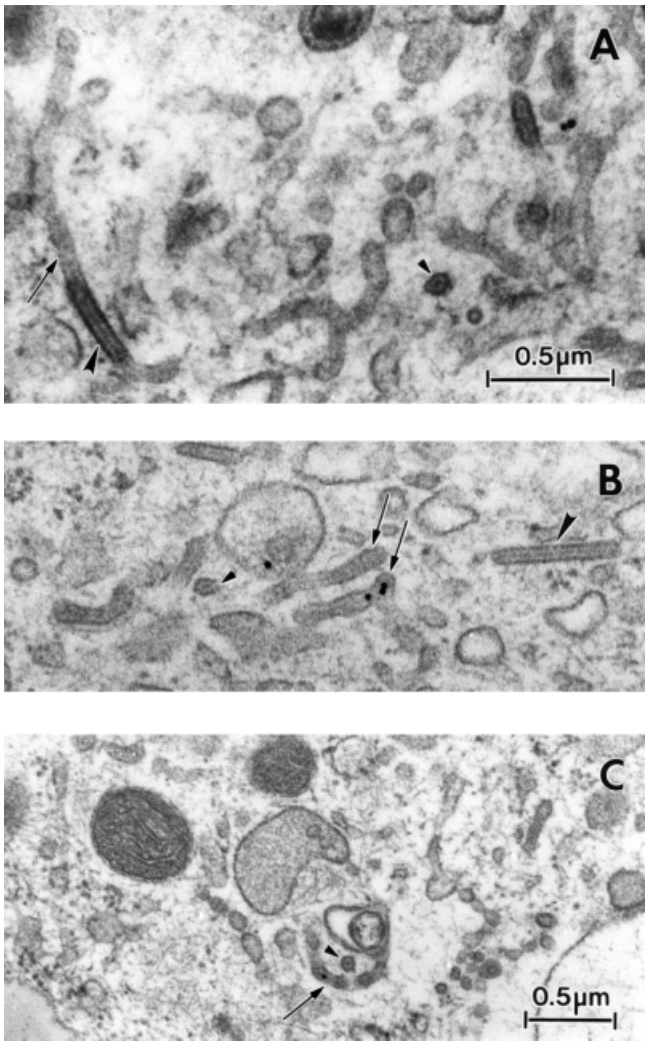

Figure 2. Scattered cored tubules are present in freshly isolated human epidermal Langerhans cells. Freshly isolated Langerhans cells were warmed to 37°C for 15 min and incubated at 37°C for 25 min with gold-conjugated Fab fragments of the anti-CD1a monoclonal antibody BL6. The three sections (*A*), (*B*), and (*C*) reveal structures that display a thin central line with no periodicity on longitudinal planes (*arrows*) but appear circular with a central dot (*thin arrowhead*) on cross-sectional profiles and thus present all the morphologic characteristics of "cored tubules". Note in (*A*) a modification in the morphology of a cored tubule, which suddenly becomes straight with the appearance of a central striated zone, then presenting the characteristic pentalaminar structure of a Birbeck granule (*arrowhead*). In (*B*) and (*C*) gold-labeling is visible on the inner central line of a cored tubule.

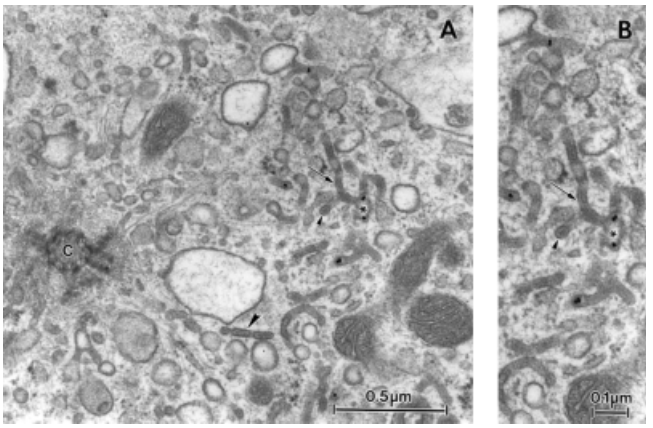

Figure 3. Freshly isolated human epidermal Langerhans cells contain networks of cored tubules. Freshly isolated Langerhans cells were warmed to 37°C for 15 min and incubated at 37°C for 25 min with gold-conjugated Fab fragments of the anti-CD1a monoclonal antibody BL6. The branched tubules visible in (*A*) in the cell center near the centriole (*C*) display all the morphologic characteristics of "cored tubules": a thin central line with no periodicity on longitudinal sections (*arrows*) and a central dot (*thin arrowhead*) on cross-sectional profiles. Note the presence of (i) a Birbeck granule (*arrowhead*) radiating from the centriole and (ii) a gold-labeled vacuolar endosome (*) in the center of the cored tubule network. (*B*) shows a higher magnification of the same network.

Langerhans cells. Cored tubules are tortuous rod-shaped structures with an inner central line lacking periodicity, which when sectioned transversely appear circular with a central dot. These tubules may be interconnected with structures known to belong to the early endosomal pathway (vesicular/vacuolar early endosomes and Birbeck granules) and can be immunolabeled with the anti-CD1a monoclonal antibody BL6-Au and the anti-Langerin DCGM4-Au, but only at 37°C. The dependence of this labeling of the cored tubules on the temperature conditions bears a resemblance to the traffic of CD1a molecules and Langerin, which permanently recycle between the early endosomal compartments and the cell surface of Langerhans cells in a temperature-dependent manner (Salamero *et al*, 2001; McDermott *et al*, 2002). Thus, at 19.5°C two markers of the early endosomal pathway of Langerhans cells, the anti-CD1a monoclonal antibody BL6 and the anti-Langerin monoclonal antibody DCGM4, progress from the cell surface to the $EEA1^+/Rab5^+$ early sorting compartments, where they accumulate. Only when the temperature is raised to 37°C do they leave the early sorting compartments and gain access to the $Rab11^+$ early recycling compartments, which in Langerhans cells include the Birbeck granules. Therefore, the fact that cored tubules can be labeled with BL6-Au only when the cells are incubated at 37°C strongly suggests that these structures belong, just like Birbeck granules, to the recycling compartments of Langerhans cells.

A relationship between cored tubules and early endosomal compartments is further supported by the findings of Valladeau (2000). This author reported that a point mutation in the proline-rich intracellular motif of Langerin (WPREPPP → WIREPPP) leads to the appearance, in 10%–20% of transfected murine fibroblasts, of authentic networks of cored tubules linked to multivesicular compartments. This proline-rich motif is known to interact with the SH3 domain proteins found in adaptors (Valladeau *et al*, 2000). Hence, the appearance of cored tubules, at the expense of tubular recycling endosomes, could be related to interactions between Langerin and adaptors in the recycling compartment. Identification of these adaptors should help to better define the nature of such interactions between Langerin and molecules known to be involved in intracellular sorting. The absence of any connection of cored tubules with the cell membrane would nevertheless suggest that these adaptors should be located only in the cytosol surrounding the recycling compartment.

Cored tubules would thus appear to result, like Birbeck granules, from interactions involving a single molecular species, Langerin. These interactions would occur in the recycling compartment where Langerin accumulates. The fact that an intradermal injection of saline solution led to the appearance of both cored tubules and Birbeck granules in murine cells "related" to Langerhans cells and located in the dermis and its draining lymph nodes suggests that activation of these cells could modulate the distribution of Langerin and regulate its traffic, in particular through the recycling compartment. This would explain the observations of Kolde and Knop (1987) and Hanau *et al* (1989), who reported that application of a hapten to the skin of, respectively, mice or guinea pigs induced activation of epidermal Langerhans cells and the appearance in their cytoplasm, during the hour following application of the hapten, of numerous Birbeck granules.

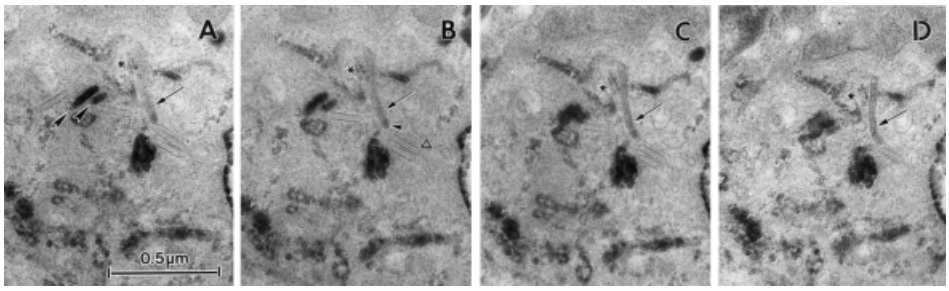

Figure 4. BFA induces fusion of cored tubules with vacuolar endosomes. Langerhans cells were incubated at 37°C for 30 min with BFA (10 µg per ml) and then for 60 min with BFA (10 µg per ml) and HRP (10 mg per ml). On thick sections (*A–D*) of the endosomal system, the connection between an HRP-positive "cored tubule" (*arrow*) and a vacuolar endosome (*) is viewed from different angles. Thus, one can see in (*B*) on a cross-sectional profile the extremity of the cored tubule (*thin arrowheads*), which appears circular with a central dot, whereas in (*D*) the angle of observation reveals a thin central line running along the tubule (*arrow*). In (*A*) the presence of two HRP-positive Birbeck granules (*arrowheads*) should be noted, together with four unlabeled Birbeck granules (*open arrowhead*). (*A*), (*B*), (*C*), and (*D*) are at the same magnification. (*A*, tilt −15°; *B*, tilt 0°; *C*, tilt +15°; *D*, tilt +35°).

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